ORIGINAL RESEARCH ARTICLE

Determination of microbial metagenomic markers of Type 2 *Diabetes Mellitus* (T2DM) in patients visiting South C Health Centre in Nairobi Kenya

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ABSTRACT

Disturbances in the gut microbiota have been associated with the onset and progression of Type 2 *Diabetes Mellitus* (T2DM). This study aims to the determination of Microbial Metagenomic Markers of Type 2 *Diabetes Mellitus* (T2DM) in Patients Visiting South C Health Centre in Nairobi Kenya. The study adopted three basic objectives, namely, types and abundance of Bacteria Colonizing the Gut of T2DM, Pre-Diabetic, and Non-Diabetic Patients Visiting South C Health Centre, T2DM metagenomics markers based on the identified genera and abundances of bacteria and correlation between metagenomic markers of T2DM, prediabetes and non-diabetic subjects and their clinical manifestations. The data used was collected from South C Medical Center in Nairobi, Kenya. The total target population was 79 persons in a cross-sectional metagenomic study which profiled the types and abundances of gut bacteria in 33 T2DM and 13 prediabetic Kenyan volunteers and compare these with the profiles of 33 Kenyans without diabetes. Postprandial random blood sugar (RBS) measurements were used to group the participants. Fecal samples were collected and subjected to 16S V5-V6 rRNA gene sequencing. Reads were analyzed using MOTHUR v. 1.39.1 and the SILVA reference dataset. Alpha and beta diversity and tests of significance were calculated using the MOTHUR Software. Samples from all the groups showed marked dysbiosis characterized by the high abundance of *proteobacteria*, low levels of bacteroidetes, and a high F/B ratio. The T2DM group had high levels of *Firmicutes* and *Actinobacteria* compared to the non-diabetic and prediabetic groups. Putative metagenomic markers of T2DM identified include elevated levels of *Firmicutes* and *Actinobacteria*, elevated F/B ratio, and high alpha diversity in T2DM (Chao 1 -value: 0.019888; [Kruskal-Wallis] statistic: 7.8353) and significant abundance of *Escherichia shigella* (p value=0.000588, FDR=0.004706). It was suggested that high levels of *Escherichia Shigella* in T2DM patients contribute to the progression of T2DM disease through the production of bacterial endotoxins leading to chronic systemic inflammation. High levels of opportunistic pathogens such as *Escherichia Shigella*, and *Kluyvera* in the T2DM group support the observation that monitoring the gut microbiome may be a useful strategy in monitoring and managing bacterial infections in the diabetic people.

1.0 Introduction

Type 2 *diabetes mellitus* (T2DM) is a chronic debilitating condition whose main features include hyperglycemia, insulin resistance, abnormal lipid, carbohydrate, and protein

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metabolism, and progressive decline leading to microvascular complications such as retinopathy, neuropathy, and nephropathy (Kahn, *et al*., 2013).

In Kenya, the prevalence of T2DM is estimated to be 5.3% (Ayah, *et al*., 2013). Known risk factors for T2DM include a sedentary lifestyle, poor diet, obesity, old age, family history, race, and genetics. These all factors affect the normal microbial gut flora so altered gut flora through the PAMPS and DAMPS ligate RRP like Nod and Toll-like receptors and causes dysregulated cytokines and chemokines due to this causes under-regulation of insulin receptors that is protein kinase and decrease anabolism and increase catabolism causing dyslipideamia which cause Microangiopathy of vessels ultimately lead to irreversible organes damage.

This study helped in reducing insulin resistance by altering the gut microbes. Diabetes can be classified into Diabetes 1 and Diabetes 2. Diabetes 1 is an autoimmune disease caused by B & T cells and it is often occurring in children below 18. It is also called insulin-dependent diabetes and it is always the HLA gene and its prevalence in 5-10% globally. While Type 2 is in 90-95% population globally and it has multi-factorial origins like age, race, geographical distribution, genetic (autosomal dominant trait), obesity, and diet all affect normal gut flora.

Previous metagenomic studies have identified bacteria such as *Faecalibacterium*, *Akkermansia* and *Roseburia* have been negatively associated with T2DM means the lower the amount of these bacteria causes the unabated progression of T2DM and higher the amount of these bacteria severity of T2DM can be plummeted. Conversely, high abundances of *Ruminococcus*, *Fusobacterium*, and *Blautia* species are positively associated with T2DM2 (Vallianou et al., 2019). These bacterias contribute to disease by increasing insulin resistance, reshaping the intestinal barrier, and altering host metabolism and signaling pathways.

2.0 Materials and methods

This study is cross-sectional in South C Medical Centre Nairobi Clinic, Nairobi. Collection of 33 diabetic patients, 33 normal persons without diabetes, and 13 pre-diabetic those sugar varies postprandial (PP) 7-8 mm per liter. After the collection of the fecal sample from them, an analysis of the sample was done at the Bioinformatics Institute of Kenya (KIBS). Three tools in this study were used; Molecular tools, Bioinformatics tool, and Statistical Module/tools.

In molecular tools, DNA extraction from all samples: diabetic, pre-diabetic, and normal then PCR using in universal primer targeting conserved sequence 16S RNA with hypervariable reasons (V1-V6).

Sequencing of amplified products is then conducted followed by bioinformatics analysis using Minion Nanopore Sequencing Machine. Alignment was done by SILVA software using a reference sequence of the Ecoli genome taken from NCBI. Statistical analysis was done by MOTHUR software.

3.0 Results

3.1 Percentage of abundance of bacterial phyla

OTUs (Operational Taxonomic Unit) were generated by MOTHUR software and analysis were

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done both by Descriptive and by Inferential Analysis. Percentage of abundance of bacterial phyla shown table 1

Table 1

	Percent Abundance (%)		
	Diabetic	Normal	Prediabetic
Proteobacteria	89.68	98.63	0.65
Firmicutes	9.25	0.98	0.28
Actinobacteria	1.08	0.39	0.07

Firmicutes and *Actinobacteria* are abundantly present in diabetics as compared to normal and pre-diabetic. *Proteobacteria* are abundantly present in diabetic and normal than pre-diabetic.

Further analysis of bacteria at the genus level was done which is shown in table 2

Table 2

	Diabetic %	**Normal %**	**Prediabetic %**
Bifidobacterium	23.8	9.5	66.7
Enterobacteriaceae_unclassified	60.3	38.2	1.5
Enterococcus	34.3	4.3	61.4
Escherichia_Shigella	63.6	34.7	1.7
Klebsiella	28.2	51.4	20.4
Kluyvera	21.1	78.9	0.0
Salmonella	16.3	61.9	21.8
Staphylococcus	51.4	5.4	43.2

Bifidobacterium, Enterococcus, and *Staphylococcus* are less abundant in normal compared to diabetic and pre-diabetic sample

Escherichia_Shigella and *Enterobacteriaceae_unclassified* are dominant bacteria in diabetic as compared to normal and pre-diabetic. The same is depicted in bar chart figure 1.

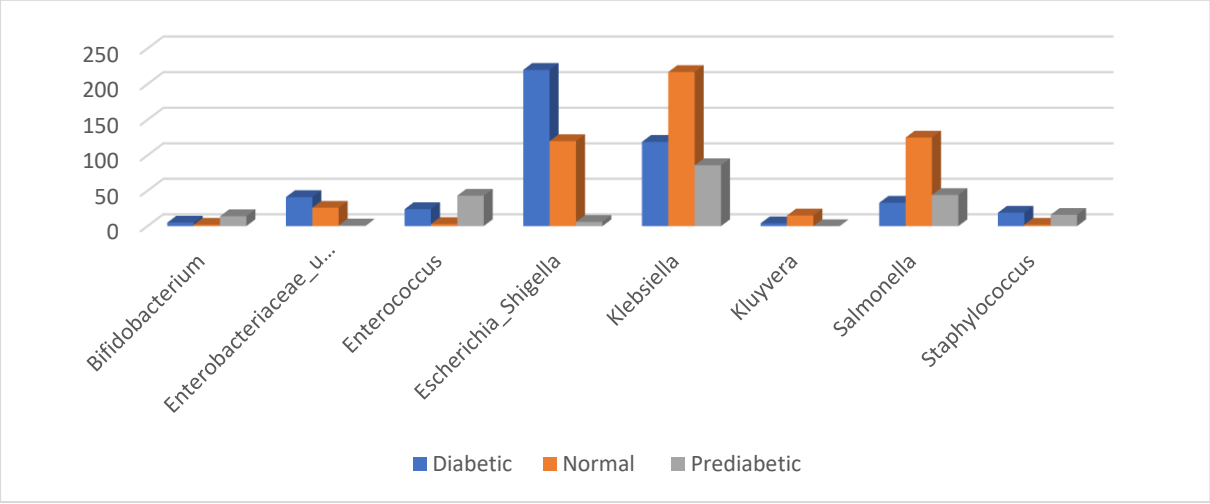

Figure 1

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Alpha diversity is a species richness (how many microbes) in each sample; diabetic, pre-diabetic and normal. Alpha diversity is shown by the Rarefaction curve shown in figure 2 Diabetic group was richer than the pre-diabetic and normal groups. The richness of a species in diabetic shows its diversity, density, and activity of gut bacteria. This shows dysbiosis in the diabetic sample.

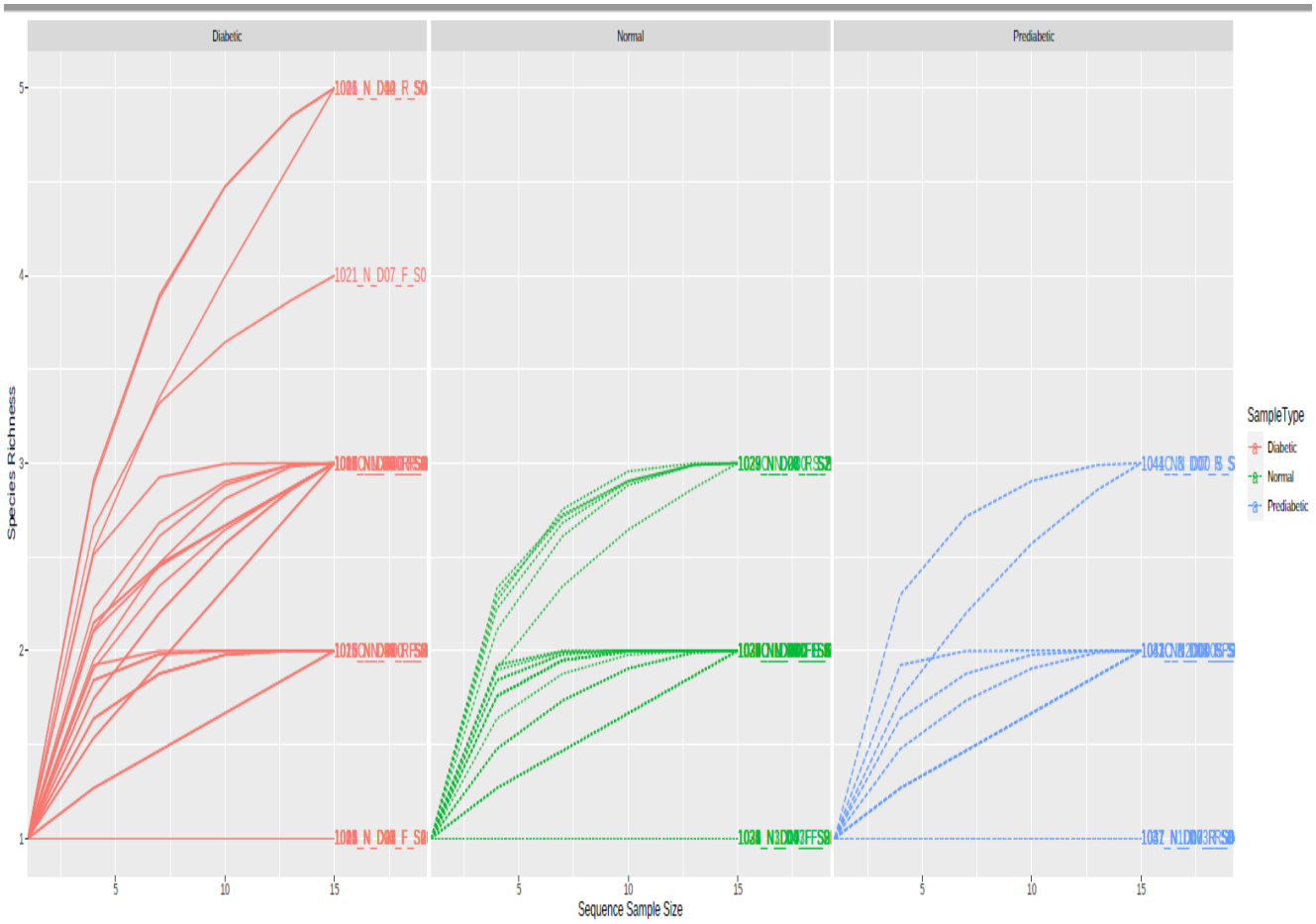

Figure 2

Diversity was calculated using the Chao 1 index and significant differences were ascertained using the non-parametric Kruskal Wallis/Mann Whitney test. The diabetic samples were significantly more diverse than the non-diabetic and prediabetic samples (Chao 1 -value: 0.019888; [Kruskal-Wallis] statistic: 7.8353).

Beta diversity is a measure of similarity or dissimilarity between samples of diabetic, pre-diabetic, and normal and matrix distances were measured by Euclidean distance between samples as shown in figure 3 Beta diversity was calculated using the Permutational MANOVA (PERMANOVA) test. The beta diversity is statistically significant [PERMANOVA] F-value: 4.1884; R-squared: 0.099279; p-value < 0.002). The chart on the right is the principal coordinate analysis (PCoA) plot.

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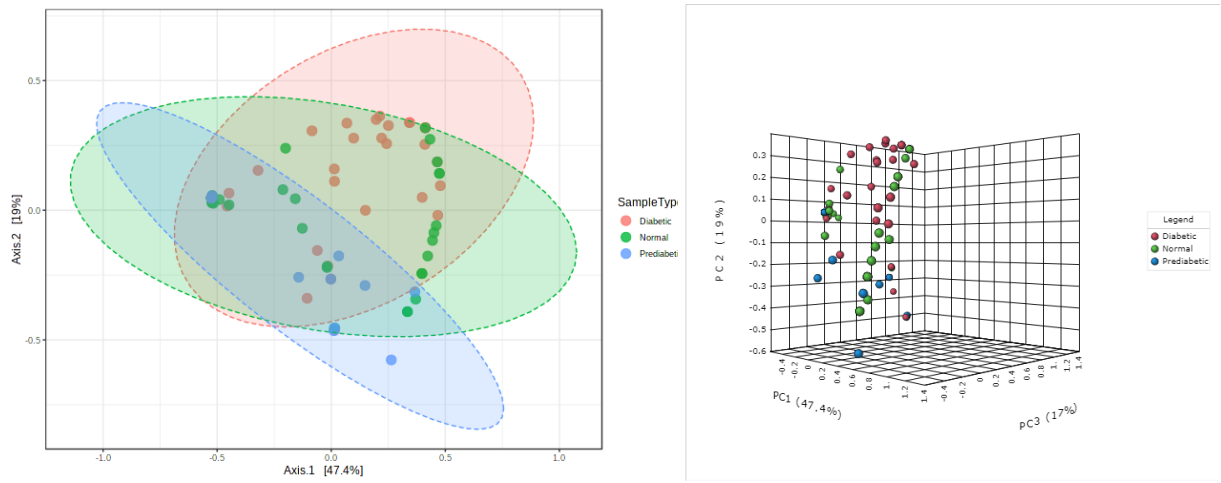

Figure 3. Beta Diversity and Principal Coordinate Anylysis (PCoA)

OTU 000001 was a statistically significant bacteria genera of *Escherichia_Shigella.*
The LDA threshold for the score was 2. OTU000001 is significantly abundant in the diabetic group compared to the normal and prediabetic groups (p value =0.000588, FDR=0.004706). All other OTUs were not significantly abundant in any group. These OTUs are shown in table 3.

Table 3: Lefse values testing significance for abundant OTUs

	P values	FDR	Statistics
Otu000001	0.000588	0.004706	14.877
Otu000004	0.089748	0.27498	4.8215
Otu000003	0.12563	0.27498	4.1488
Otu000007	0.13749	0.27498	3.9684
Otu000020	0.23729	0.37966	2.877
Otu000006	0.47369	0.63158	1.4944
Otu000002	0.78462	0.89671	0.48511
Otu000033	0.94045	0.94045	0.1228

Statistically significant OUT 000001(*Escherichia_Shigella.*), box plot was drawn which is shown in figure 4. In diabetic both the upper quartile and lower quartile are most conspicuous and there is a median visible and upper and lower whiskers are clear than in normal and pre-diabetic.

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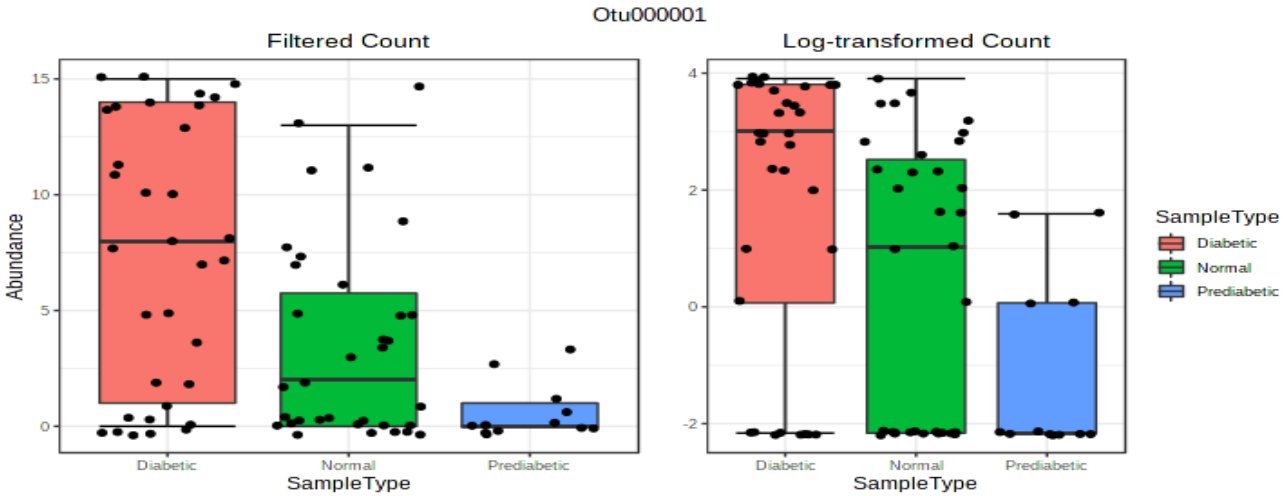

Figure 4. Box plot of OTU 000001(Escherichia_Shigella)

3.2 Random forest (RF) analysis of the three groups (diabetic, non-diabetic (normal) and prediabetic datasets)

Figure 5 shows RF is an ensemble learning method with high utility in classification. The RF used the diabetic, non-diabetic (normal), and prediabetic datasets. The prediabetes group had a relatively high error rate compared to the other groups. The normal group had the lowest error rate.

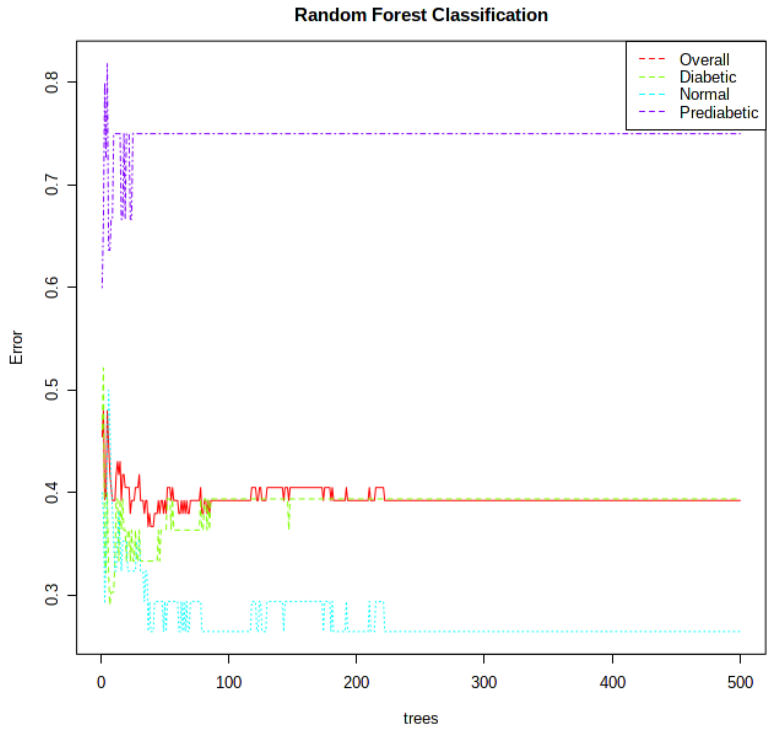

Figure 5: Random Forest (RF) classification of the diabetic, prediabetic and normal groups.

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The robustness of the RF classification was assessed using the out of bag (OOB) error rate. The OOB rate is 0.392. The class error for the prediabetic group was highest at 75% and lower for diabetic (39.4%) and Normal (26.5%).

	Diabetic	Normal	Pre-diabetic	Class.error
Diabetic	20	10	3.0	0.394
Normal	8	25	1.0	0.265
Pre-diabetic	1	8	3.0	0.75

3.3 Mean decrease accuracy (MDA) analysis of diabetic, pre-diabetic and normal groups' OUT.

The higher value of Mean Decrease Accuracy (MDA), the higher the importance of the variable in the model. Important predictors of diabetes are OTU000001, OTU000006, OTU000003, and OTU000020. Important predictors of normal are OTU000004 and OTU000002. Important predictors of prediabetes are OTU000007. The Mean Decrease Accuracy (MDA) plot expresses how much accuracy the model losses by excluding each variable. Mean accuracy plot shown in figure 6 and table 4 shows important Predictors of the Diabetic, Pre-diabetic, and normal groups.

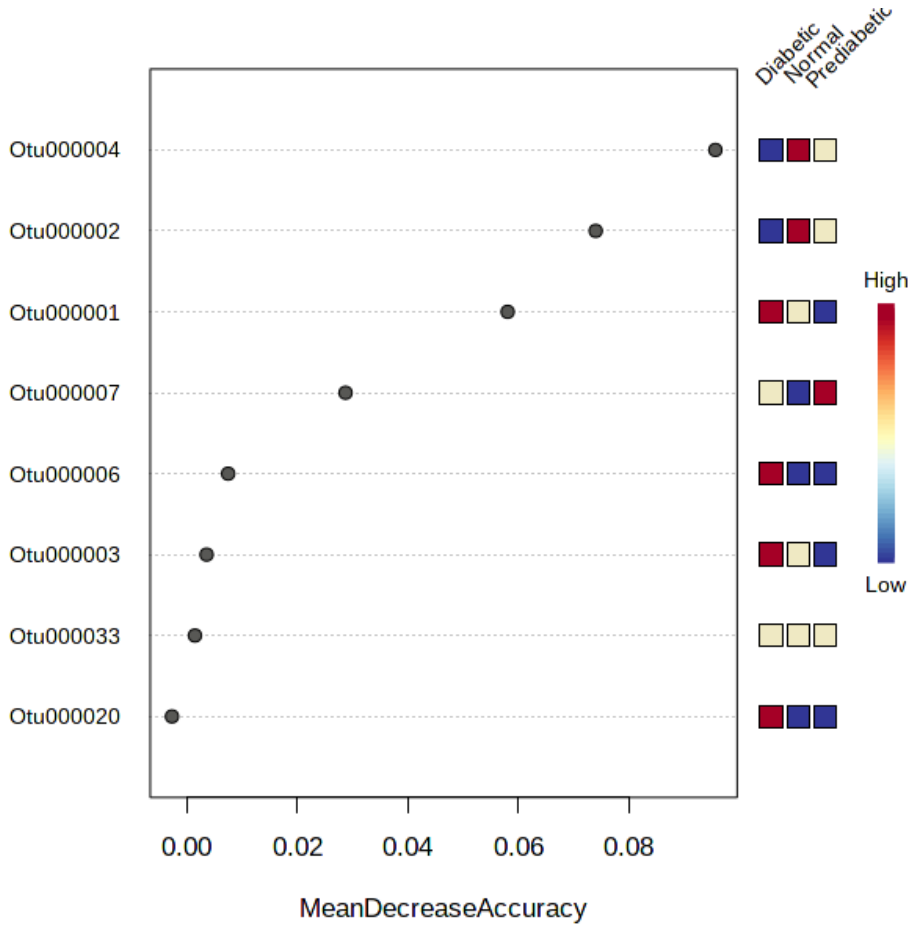

Figure 6: Mean Decrease Accuracy (MDA) plot

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Table 4: Important Predictors of the Diabetic, Pre-diabetic, and normal groups

Predictor	Group	Phylum	Family	Genus
OTU000001	Diabetes	*Proteobacteria*	*Enterobacteriaceae*	*Escherichia_Shigella*
OTU000003	Diabetes	*Proteobacteria*	*Enterobacteriaceae*	*Enterobacteriaceae_unclassified*
OTU000006	Diabetes	*Proteobacteria*	*Enterobacteriaceae*	*Kluyvera*
OTU000020	Diabetes	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus*
OTU000004	Normal	*Proteobacteria*	*Enterobacteriaceae*	*Salmonella*
OTU000002	Normal	*Proteobacteria*	*Enterobacteriaceae*	*Klebsiella*
OTU000007	Prediabetes	*Firmicutes*	*Enterococcaceae*	*Enterococcus*

Table 4 shows Important Predictors of the Diabetic, Pre-diabetic, and normal groups

4.0 Discussion

In this study, 16s rRNA sequencing on fecal samples were obtained from 33 diabetic, 33 non-diabetic, and 13 prediabetic Kenyan patients. The main objective was to profile the different types and abundances of bacteria in each group and determine metagenomic markers of T2DM and prediabetes in Kenyan patients. To determine the metagenomic markers of T2DM, analysis of sequencing reads, to determine the following five parameters within and between the groups: relative bacterial abundance, species diversity, significantly abundant genera, and important predictors were taken.

4.1 Types and abundance of bacteria colonizing the gut of T2DM, pre-diabetic, and non-diabetic patients visiting South C Health Centre

Relative bacterial abundance describes the percentages of specific bacteria making up the entire microbiome under study. The study findings show that the dominant phyla in the three groups under study were *Proteobacteria* (89%), *Firmicutes* (9%), and *Actinobacteria*. (1.8%). *Proteobacteria* was most dominant in the non-diabetic group (98.6%) and least dominant in the prediabetic group (0.65%). *Firmicutes* were more than 20 times more abundant in the diabetes group (9.25%) compared to the prediabetes group and nine times more abundant in the diabetes group compared to the non-diabetic group (0.98%). *Actinobacteria* was also more abundant in diabetes (1.08%) than in the normal (0.39%) and prediabetic groups (0.07%). The abundance of *Bacteroidetes* was low in all the 3 groups.

Proteobacterial species are known to be archetypal signatures of microbial dysbiosis (Shin *et al*., 2015). The normal gut microbiome of healthy people comprises the following 4 phyla: *Firmicutes* (64%), *Bacteroidetes* (23%), *Proteobacteria* (8%), and *Actinobacteria* (3%) in that order. *Verrucomicrobia* and *Fusobacteria* are also present but in smaller quantities (Turnbaugh *et al*., 2007; Blaut, 2013).

Study findings are inconsistent with other findings as they indicate marked dysbiosis across all three groups. Differences with other findings also include markedly elevated levels of *proteobacteria* and reduced levels of *Firmicutes* and *Bacteroidetes*. This was observed not only in the diabetic and prediabetic groups but also in the non-diabetic group. The findings may suggest that relatively abundant *Proteobacteria* and reduced *Firmicutes* and *Bacteroidetes* levels may be used as metagenomic markers of T2DM. However, dysbiosis in the non-diabetic

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samples suggests that such an interpretation be used with caution. These unusual findings can be attributed to the use of patient samples.

Normal values reported in our study are very different from those reported in the normal human gut microbiota. Several studies have reported significantly lower levels of *Firmicutes* but significantly higher levels of *Bacteroidetes* and *Proteobacteria* in T2D. This results in a low F/B ratio in the gut of people with T2D (Schwiertz A, et al. 2009). A high F/B ratio in T2D has also been noted following reduced *Bacteroidetes* with elevated levels of *Firmicutes* and *Proteobacteria* (Sedighi *et al*., 2017; Komaroff, 2017). A high F/B ratio is a marker of elevated plasma glucose (Larsen *et al*., 2010). No significant differences have been reported in other studies evaluating the gut microbiota in T2DM patients (Lambeth *et al*., 2015). This study's findings observed an elevated F/B ratio. This finding is consistent with the results of other studies reported elsewhere (Zhao *et al*., 2019; Sedighi *et al*., 2017; Komaroff, 2017).

Differences between this study's findings and those reported may be attributed to the presence of opportunistic pathogens in the population under study. Indeed, this study analysis of the dominant genera demonstrated that the dominant genera were *Escherichia Shigella* (34.3%), *Klebsiella* (32.8%), and *Salmonella* (14.2%). All these are pathogenic organisms. *Kluyvera* and staphylococcus were also present in abundant amounts. The presence of these organisms in abundant quantities suggests that sample patients had other pre-existing conditions. It may also serve as a pointer to the observation that people with diabetes are prone to infections. This adds weight to the speculation that the observed abundances at phyla and genus levels are not accurate reflections of the gut microbiota but are confounders that skewed our data hence the unusual findings being reported here. Further test whether these bacteria were significantly elevated in any of the three groups. Study findings show that *E. shigella* was significantly higher in the diabetic group ((p value=0.000588, FDR=0.004706). the rest of the genera were not significantly abundant in any of the three groups. A recent study by Maskarinec *et al* (2021) found elevated levels of E. shigella in patients with T2DM. A high abundance of *E. Shigella* is an endotoxin-producing bacteria and is thought to enhance chronic systemic inflammation and T2DM disease. Additionally, elevated levels of *E. Shigella* have been associated with the use of metformin in T2DM patients (Mascarinec *et al*., 2021).

Previous metagenomic studies have identified several bacteria that are significantly elevated in T2DM. *Faecalibacterium, Akkermansia, and Roseburia* have been reported in diabetic patients and contribute to disease by increasing insulin resistance, reshaping the intestinal barrier, and altering host metabolism and signaling pathways. Conversely, high abundances of *Ruminococcus, Fusobacterium,* and *Blautia* species are positively associated with T2DM2 (Vallianou *et al*., 2019). This study did not report any of these bacteria.

Alpha Diversity was calculated using the Chao 1 index and significant differences were ascertained using the non-parametric Kruskal Wallis/Mann Whitney test. Observations were that the diabetic samples were significantly more diverse than non-diabetic and prediabetic samples (Chao 1 -value: 0.019888; [Kruskal-Wallis] statistic: 7.8353). Having determined that diabetic samples were significantly richer, the study set out to determine the specific bacterial groups associated with this richness and their distribution. Observation was made

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As with the abundance data, caution in interpreting this finding is needed. Besides the confounding variables that have already been mentioned, studies have found an inverse association of alpha diversity with T2DM and metformin use. In this study, no control for metformin use or use of any other medication was done therefore the findings could not be correlated to medication nor were the findings conclusively interpreted in the absence of this data.

4.2 T2DM metagenomics markers based on the identified genera and abundances of bacteria

Elevated levels of *Firmicutes* and *Actinobacteria* may be used as metagenomic markers of T2DM. These findings suggest that diabetic samples can be distinguished from prediabetes samples by the high abundance of *Proteobacteria*, *Firmicutes*, and *Actinobacteria* species in the former group. An elevated F/B ratio in diabetic samples was observed and may be used as a metagenomic marker of T2DM. Another putative T2DM metagenomic marker was the high alpha diversity of T2DM - the diabetic samples are significantly more diverse than non-diabetic and prediabetic samples (Chao 1 -value: 0.019888; [Kruskal-Wallis] statistic: 7.8353). High alpha diversity may be used as a metagenomic marker of T2DM.

Measurement of species diversity within and between groups is used to evaluate microbiomes. Higher-level species measurements include alpha and beta diversity. Alpha diversity measures the richness of species in one particular group or sample while beta diversity evaluates the similarity or dissimilarity of 2 or more given communities. In this study, we applied alpha diversity measurements to determine the numbers and distribution of bacteria in diabetes, non-diabetes, and prediabetes groups respectively. Beta diversity measurements were also used to find out if bacteria in diabetes, non-diabetic and prediabetic were evenly distributed across the groups or if the microbial diversity in any of the 3 groups was significantly different from each other group, and if there were any changes in species diversity between the 3 groups. The expectation was that any significant differences in alpha or beta diversity differences in and between groups would be proposed as metagenomic markers for T2D. Reductions in diversity and or increase in levels of bacteria such as *Proteobacteria* are common markers of dysbiosis and the use of alpha and beta measurements was also done to determine if dysbiosis is a characteristic feature of T2D and prediabetes in Kenyan patients as reported in the literature in other jurisdictions (Qin *et al*., 2012)

Dominant genera were identified as *klebsiella*, *Escherichia-shigella*, *Salmonella*, and *Enterococcus.*

4.3 The correlation between metagenomic markers of T2DM, prediabetes and non-diabetic subjects and their clinical manifestations

Study findings suggest that T2DM metagenomic markers proposed here can be correlated to clinical manifestation of T2DM in 2 ways. First, *Escherichia_Shigella* is known to produce pro-inflammatory endotoxins. A constant feature of T2DM disease is constant low-grade inflammation. The proinflammatory endotoxins associated with high abundance of *Escherichia_Shigella* in T2DM patients may be partly responsible for the chronic systemic inflammation in these patients. This observation seems to support findings by Maskarinec *et*

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al (2021) who recently reported elevated levels of *E. shigella* in patients with T2DM and associated this abundance with chronic systemic inflammation in T2DM disease (Mascarinec *et al*., 2021). The implication is that screening and treatment of *Escherichia_Shigella* infestation may perhaps slow down the low-grade inflammation hence assist in improving outcomes of T2DM disease.

In the second instance, we observed high levels of opportunistic pathogens such as *Escherichia_Shigella,* and *Kluyvera* in the T2DM group. Many studies have previously shown that people with T2DM are more susceptible to infections compared to non-diabetic persons. The clinical implication is that screening of the gut microbiome may be a useful strategy in monitoring and managing bacterial infections in people with T2DM.

5.0 Conclusions and recommendations

5.1 Conclusions

This study sought to find the metagenomic markers of T2DM in a cohort of Kenyan patients. Using 16s rRNA sequencing, 79 samples were investigated from diabetic, non-diabetic, and prediabetic patients.

Firmicutes were nine times more abundant in diabetic compared to non-diabetic groups. Actinobacteria was also most abundant in the diabetic group compared to the non-diabetic and prediabetic groups. Samples from all the groups showed marked dysbiosis characterized by the high abundance of proteobacteria, low levels of *bacteroidites,* and a high F/B ratio. Putative metagenomic markers were identified as follows:

i. High levels of *Escherichia_Shigella may be* used as metagenomics markers of T2DM.
ii. Elevated levels of Firmicutes and Actinobacteria may be used as metagenomics markers of T2DM.
iii. Elevated F/B ratio in diabetic samples was observed and may be used as a metagenomics marker of T2DM.
iv. The diabetic samples are significantly more diverse than non-diabetic and prediabetic samples (Chao 1 -value: 0.019888; [Kruskal-Wallis] statistic: 7.8353). High alpha diversity may be used as a metagenomics marker of T2DM.

5.2 Recommendations and future prospects

i. Metagenomics markers in type 2 *Diabetes Mellitus* were never done in Kenya; the first study of this type is conducted for the first time in Kenya.
ii. From this study, it is recommended action should be taken by a competent authority like in Kenya by PPB (Pharmacy and poison Board) and MOH to put in place and consider fecal implantation on T2DM from Normal gut flora to abate and mitigate the complication from T2DM to prevent irreversible damage of vital organs (H Wang 2019 *et al,* Fecal microbiota transplantation and Lee *et al*, 2018 Fecal microbiota transplantation is a method to treat T2DM.)

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6.0 Acknowledgements

6.1 Presentation of the study, findings, and a portion of the work

A portion of the work done was presented in seminar at The Kenya Medical Research Institute (KEMRI) on 5th April 2022 Nairobi, Kenya.

6.2 General statement

For the success of "Determination of Microbial Metagenomic Markers of Type 2 *Diabetes Mellitus* (T2DM) in Patients Visiting South C Health Centre in Nairobi Kenya" research project supervision was necessary for its completion. Prof Johnson Kinyua P.H.D Chairman Department of Biochemistry, JKUAT, Kenya and Dr. James Kimotho P.H.D. Head Department of Innovation and Technology, KEMRI, Nairobi, Kenya, author is grateful to both supervisors.

The author is also appreciative to the 79 patients who signed the Informed Consent about the samples and confirmed their willingness to engage in this research and evaluation at the South C Medical Centre in Nairobi. KIB (Kenya Institute of Bioinformatics), where the analysis was carried out, deserves special mention.

6.3 Declaration of interests

Researcher did not receive any financial relationships construed as a potential conflict of interest.

Ethical approval for Research was on 24th June 2021, Reference number being JKU/2/4/896B and issued by Jomo Kenyatta University of Agriculture and Technology(JKUAT), Nairobi, Kenya ERC.

The current project is in completion of the M.Sc. Molecular Medicine degree requirement. The manuscript studies were organized in a chosen manner based on their relevance to the subject matter and predicted work quality, rather than being exhaustive. This article's opinions, assessments, knowledge, and conclusions are exclusively those of the author.

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